Fortuitous insights into the ecology of a recently charted deep-sea hydrothermal vent, using snails' feet.

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Abstract

Here we present the results from a shotgun sequencing effort on foot tissues from a deep-sea hydrothermal vent endemic limpet. We present the complete mitochondrial genome of the hydrothermal vent endemic gastropod *Peltospira smaragdina* (Gastropoda, Peltospiridae) is presented here for the first time. This species is characteristic of circa-Azores hydrothermal vent ecoregion and provides a candidate environmental DNA (eDNA) indicator of active hydrothermal vent sites. The results also suggest that the epilithic biofilm on the newly discovered Moytirra hydrothermal vents is dominated by *Sulfurimonas* –like microbes and corresponds with similar studies on hydrothermal hosted microbial communities. The association between *Peltospira* and *Sulfurimonas* is presented as potentially a holobiontic relationship, with both the snail and the microbial biofilm. We highlight the efficacy of using non-traditional sampling to develop a broader ecosystem understanding. Additionally, the complete mitochondrial genome of the hydrothermal vent endemic gastropod *Peltospira smaragdina* (Warén & Bouchet, 2001) is presented here for the first time.

Introduction

Peltospira (McLean, 1989) is a genus of small limpet-like gastropods that appear to be common at, and apparently endemic to, active deep-sea hydrothermal vents. *P. smaragdina* is the type species for the Northern Mid Atlantic Ridge (MAR). This is a gregarious genus has been observed on MAR vents sites in aggregations of between 12-50 individuals (Warén & Bouchet, 2001). Three other species in the genus *P. operculate, Peltospira lamellifera* and *P. delicata,* occupying similar ecological niches, have been observed at hydrothermal vent sites in the East Pacific (Shank, 1997).

Peltospira have not been observed hosting microbial chemosymbionts (Warén & Bouchet, 2001). Instead, *Peltospira* appear to graze on bacterial mats using the hooked marginal teeth on their radula to scrape away the biofilm (Okutani *et al.,* 1993). The nature of this biofilm is currently unknown. However, *P. smaragdina* have only been recorded from deep-sea hydrothermal vents – in association with the large biomass of fast-growing chemoautotrophic microbes that are nourished by the highly reduced hydrothermal vent fluids (Orcutt *et al.,* 2011). This line of evidence would suggest that their endemicity is driven by an association with vent biofilms.

The vent fluid, contains high concentrations of reduced sulphur compounds (e.g. elemental sulfur, thiosulfate, sulphide, sulphite) that directly determine the physical and chemical environment of the vent structures and, by extension, the associated microbial biofilm (Reveillaud *et al.,* 2016). This is reflected in the observation of Epsilonproteobacteria (e.g. *Sulfurospirillum, Sulfurimonas* and *Sulfurovum)* as characterising primary producers (Perner *et al.,* 2013). These sulphur oxidising microbes utilise the reduced sulphur compounds as electron donors - a locally superabundant energy source.

Here we present the results of a next generation sequencing (NGS) effort using the foot tissues of a putative *Peltospira spp.*. This effort was initially to develop microsatellite markers for a demographic study, the NGS data set was further interrogated giving the insights presented here. Here, we present the full mitochondrial DNA sequence of this species, which has potential applications in the identification of eDNA biomarkers for active hydrothermal vents. Whist these species-specific results may only interest a small select audience, the paper also provides some unexpected, derived insights into the ecology of a poorly studied deep-sea limpet and of the microbial biofilms found at active hydrothermal vent sites. This paper highlights the potential of data mining NGS results for unexpected and fortuitous insights, particularly in deep-sea settings.

Methods

Peltospira samples were collected from the Moytirra hydrothermal vent site is situated on the Mid Atlantic Ridge (45°48 N, -27°85 E) in 3000 m water depth in 2011 using the *ROV Holland I* aboard the *RV Celtic Explorer* (for a detailed site description see: Wheeler et al., 2013). The Peltospira were in aggregations far denser than previously recorded with patches of up to 50,000 specimens per square meter (see Wheeler *et al*. 2013). Individuals were preserved in molecular grade ethanol. Foot tissues were dissected and cleaned by rinsing in double distilled water to remove possible contaminants. DNA was extracted using DNeasy

Blood and Tissue Kit (Qiagen, Inc. Valencia, CA). DNA concentrations were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and standardised to a concentration of 50 ng/µl. Normalised DNA extracts from five individuals were pooled to a final volume of 65µl at 24.6 µg/µl. For shotgun sequencing, half an Ion Torrent (318 chip) NGS run was utilised at the IGCP Genome Sequencing and Analyses Core Resource, Duke University, USA.

Contig files were de-novo assembled using MIRA. Consensus sequences were extracted from the resulting contigs and used in a local Blast search against the NCBI nucelotide database (downloaded February 2016). To confirm species identity, the generated mitochondrial genome was compared to existing mtDNA in GenBank. The mitochondrial genome was annotated *de novo* using the software package MITOS (Bernt et al., 2013).

For the biofilm investigation, contig files were de-novo assembled using Geneious 7. The Megabalst subroutine of NCBI's Blastn suite was used to identify highly similar sequences to resulting assembly consensus sequences. Obvious and contaminated hits were removed from the datasets (e.g. *Peltospira smaragdina, Salmo salar, Danio rerio,* uncultured microbes etc.). When multiple Blast hits per original sequences were recorded, hits with the highest Bit score were retained.

Results

The limpets were partitioned into discrete grazing zones at far higher concentrations (circa 50,000/m^2) than previously observed for the species (Figure 1). These zones of were delineated by lines of white anhydrite precipitation, which form in cracks in the sulphide deposits and allow for heat dissipation. The white mineralisation forms at temperatures of at least 150°C (Wheeler *et al.,* 2013). Limpets were not observed to cross these boundaries, although many were in close proximity. Potential limpet predators including crabs of the genus *Segonzacia* spp. and eelpout of the genus *Pachycara* spp. were observed in proximity to the vent chimneys (Wheeler *et al.,* 2013).

Comparison with existing *Peltospira* spp. data in Genbank, using the blastn algorithm, confirmed the specimens to be *Peltospira smaragdina*. The complete mitochondrial genome sequence of *P. smaragdina* has been submitted to GenBank (accession number KX034224). The total length of the complete mtDNA is 15,522 bp. The organisation of the genome is shown in Table 1. Mitochondrial genes are encoded on the heavy strand. In addition to gene sequences for the large and small ribosomal RNAs and 22 RNAs (tRNA), the mtDNA contains 16 protein coding gene sequences. The overall base composition of the mtDNA is 36.1% A, 15.1% C, 15.4% G, 33.4% T with 30.5% GC. Only three protein coding gene sequences contained orthodox ATG start codon, ATP6, COB and COX1. There are 35 intergenic regions varying in length from 1bp to 215bp. The lengths of the tRNA gene sequences vary from 62bp ($tRNA^{Arg(gca)}$) to 73bp ($tRNA^{Asp}$). All the tRNA genes can be folded into a typical cloverleaf secondary structure. None of the tRNA had a variable loop. The mtDNA arrangement of *P. smaragdina* is identical to the complete mitochondrial genome of the Peltospididae *Chrysomallon squamiferum* (Genbank accession number AP013032).

In total, 161 of the microbial contigs extracted from the limpets' foot tissues had matches to existing sequences in the NCBI nucelotide database. The most common matched contig was *Sulfurimonas autotrophica* (89.4%) matched with *Sulfurimonas autotrophica,* followed by *Sulfurimonas denitrificens (*7.5%) with *Sulfurovum* sp. (1.2%) and single contigs of *Brevibacillus brevis, Arcobacter* sp. L and *Oleispira antarctica* (0.6% respectively) (Table 1).

Discussion

Our results confirm that the most commonly observed limpet at Moytirra is *P. smaragdina*. This finding along with the presence of other endemic vent species (e.g. *Rimicars exoculata, Mirocaris fortunata*) support the inclusion of the Moytirra vent field as the most Northwardly extension of the circa-Azores hydrothermal vent ecoregion discovered to date (Wheeler et al., 2013). The assembly of the complete mtDNA genome for *P. smaragdina* represent novel information. These data can be used to develop species specific primers that can be used to address phylogenetic and phylogeographic questions about the species including. Further, mtDNA is the preferred target for developing environmental DNA assays due to the relatively higher abundance of mtDNA when compared to nuclear DNA in organisms. Environmental DNA promises a non-invasive sampling method and detection of *P. smaragdina* DNA in deep-sea water samples (for instance collected by CTD) could indicate that hydrothermal vents are present in the vicinity of the water sample. As such, the assembly of a complete mtDNA genome for this species will allow the development of environmental DNA as a tool for the detection of active hydrothermal vent field sites.

Each anhydrite delimited patch had approximately 50% limpet coverage – highlighting both the space required per limpet and the high productivity of a fast growing epilithic biofilm – areas little more than twice the limpets diameter were sufficient. The microbial contig data suggests that the fields were near monocultures, dominated by *Sulfurimonas*-like microbes with some *Sulfurovum, Brevibaccilus, Arcobacter* and *Oleispira*-like microbes. Grazing may promote microbial productivity through removal of unproductive components of the biofilm matrix. We hypothesise that grazing activity influences biofilm permeability, regulating microbial growth rates and competitive interactions (Skov *et al.,* 2010). Thus, limpet-biofilm interactions may have implications for hydrothermal vent ecosystem functioning. The exact nature of the biofilm-limpet interaction is beyond the scope of this study. However, given the feeding ecology of this limpet it is reasonable to infer that the *Sulfurimonas-Peltospira* association is likely to be symbiotic; presenting as a holobiont (*sensu* Margulis, 1991) that supports *Peltospiras*' observed obligate vent endemism. This is further reinforced by the presence of anhydrite seams partitioning the assemblage, which appear restrict the movement of limpets around vent field (Figure 1). Furthermore, no potential limpet predators were observed within the areas bounded by the seams, suggesting that the anhydrite may act as a barrier.

The evidence suggests that the Moytirra active vent biofilm, in association with *Peltospira smaragdina* herds, supports an ecosystem with low diversity of microbial Operational Taxonomic Units (OTUs), dominated by sulfur reducing Epsilonproteo bacteria. This reflects previous vent biofilm studies that identified, specifically, *Sulfurimonas* as characteristic of

vent microbiomes (e.g. Inagaki, 2003; Sievert *et al*., 2008; Akerman *et al.,* 2015; Perner *et al.,* 2013). This is further supported by the dissection methodology –muscle and skin tissues from the well-developed foot, not gills or intestinal tract were used for DNA extraction. Both lines of evidence support the thesis that the sequenced microbial OTU's are reflective of the external environment – the entire range data set could be considered a hologenome. We do not suggest that sampling environmental microbes from gastropod podia is a 'best-practice' protocol for understanding deep-sea ecosystems. The caveats when interpreting environmental data are too many to list. Rather, this insight highlights the benefits of a broader and multidisciplinary approach in interrogating NGS data, especially from data poor and difficult to sample ecosystems. It is likely that a wealth of data and knowledge could be gleamed from existing sample stores – reducing the requirement to disturb these ecosystems and make maximum use of the sample's value.

Deep sea hydrothermal vent microbial ecology remains understudied. This is because deep-sea sampling is expensive with limited berths available for scientists and a perceived limited value of such studies. This is especially relevant today given the imminent dawn of a deep-sea mining industry – we have no information on how the microbial community will recover from the mining process. To best manage the environmental risks associated with the mining process, a better understanding of both the macro and microbial ecosystems is required. This requires deep-sea science to be more efficient with its resources – with more data and sample sharing across disciplines (see Collins *et al*., 2013). Our study highlights the benefits of a multidisciplinary approach to sample triage and processing - the limpet samples were taken from a geological sample and our elucidation of the microbial biofilm was a fortuitous benefit from a next generation sequencing microsatellite development effort on the limpets.

208 **Figures & Tables**

209 **Figure 1.** Aggregations of *P. smaragdina* (red circle) highlighting the partitioning the limpets
210 into assemblages outlined by anhydrite (green arrow) in. The orange brown material is iron
211 oxide.

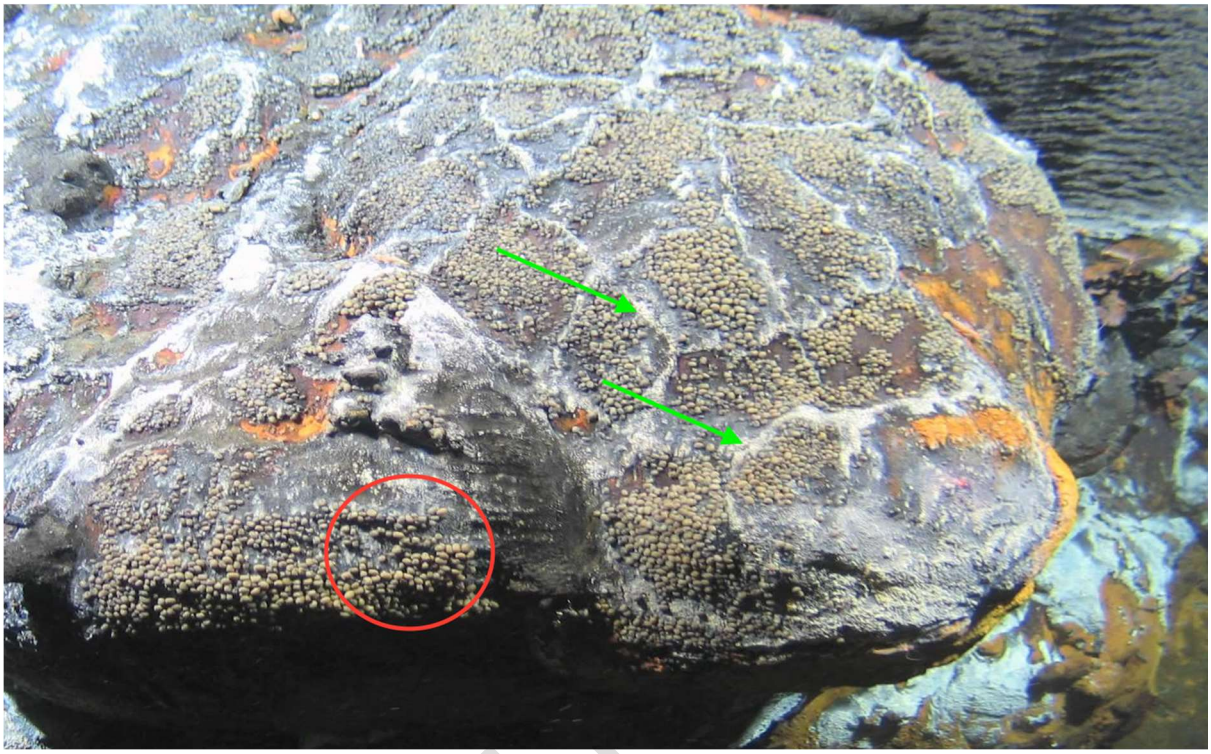

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Table1. Mitochondrial genome organization of *Peltospira smaragdina*

Gene	Start position	Stop position	length (bp)	Start codon	Stop codon	Anticodon	Intergenic nucleotides	strand
$tRNA^{Asp}$	216	288	73			UUG	215	+
$tRNA^{Ser}$	289	355	67			UCA	-	+
$tRNA^{Arg(gca)}$	357	418	62			GCA	1	+
$tRNA^{His(gua)}$	419	485	67			GUA	-	+
$tRNA^{Arg\ (ucc)}$	486	550	65			UCC	-	+
COX3	682	1332	651	GTG	TCA		131	+
$tRNA^{Lys(uuu)}$	1351	1415	65			UUU	18	+
$tRNA^{Thr(ugc)}$	1420	1486	67			UGC	4	+
$tRNA^{Cys(acg)}$	1500	1569	70			ACG	13	+
$tRNA^{Cys(guu)}$	1570	1635	66			GUU	-	+
$tRNA^{His(gau)}$	1640	1709	70			GAU	4	+
ND3	1704	2054	351	GTG	TGG		-6	+
$tRNA^{Ala}$	2065	2131	67			GCU	10	+
ND4b	2120	2176	57	ATT	TTT		-12	+
ND2	2188	3078	891	ATA	TCT		11	+
ND5b	3140	3202	63	TTG	GAT		61	+
COX1	3228	4739	1512	ATG	GAA		25	+
$tRNA^{Leu}$	4789	4854	66			GAA	49	+
$tRNA^{Pro}$	4861	4926	66			UGU	6	+
ATP6	4969	5619	651	CAT	CAT		42	-
ATP8	5655	5813	159	ATG	TGA		35	-
$tRNA^{Glu}$	5814	5879	66			GUC	-	-
COX2	5909	6586	678	ATT	TGA		29	-
ND5a	6595	8271	1677	ATT	TTA		8	-
$tRNA^{His(gug)}$	8314	8377	64			GUG	42	-
ND4a	8388	9608	1221	ATA	TGG		10	-
ND4L	9749	10018	270	TTG	TGT		140	-
$tRNA^{Thr(uga)}$	10049	10113	65			UGA	30	-
COB	10128	11258	1131	ATG	ATT		14	-
$tRNA^{Lys(uuc)}$	11261	11326	66			UUC	2	-
ND6b	11348	11575	228	TTT	CGG		21	-
ND6a	11560	11790	231	ATT	TTT		-16	-
$tRNA^{Cys(acg)}$	11837	11902	66			ACG	46	-
ND1	11946	12851	910	ATA	TTA		43	-
$tRNA^{Iso}$	12849	12915	67			UAA	-3	-
$tRNA^{Stop}$	12923	12987	65			UAG	7	-
RRNL	12963	14350	1388				-25	-
tRNA	14339	14403	65			UAC	-12	-
RRNS	14396	15272	877				-8	-
$tRNA^{Val}$	15271	15336	66			CAU	-2	-

Table 2 Contig files from the next generation sequencing effort that had matches to existing sequences in the NCBI nucleotide database

	n	*Bit average*	*Bit L*	*Bit H*	*% average*	*% L*	*% H*
Sulfurimonas autotrophica DSM 16294	144	2346	414	14475	78.5	72.5	93.1
Sulfurimonas denitrificans DSM 1251	12	965.9	464	2052	75.7	72.8	80.5
Sulfurovum sp. NBC37-1	2	4375.5	789	7962	81.3	76	86.6
Brevibacillus brevis NBRC 100599	1	1304	1304	1304	74.5	74.5	74.5
A*rcobacter sp.* L	1	412	412	412	75.4	75.4	75.4
Oleispira antarctica RB-8	1	538	538	538	74.7	74.7	74.7

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Conflict of Interest statement

The authors declare that they have no financial or equity interest in the subject matter or materials discussed in this manuscript.

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